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Bacteriophage λ *P* Gene Shows Host Killing Which Is Not Dependent on λ DNA Replication

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Bacteriophage λ, having a mutation replacing glycine by glutamic acid at the 48th codon of *cro*, kills the host under N^- conditions; we call this the *hk* mutation. In $\lambda N^-N^-c\mathrm{I}^-hk$ phage-infected bacteria, the late gene *R* is expressed to a significant level, phage DNA synthesis occurs with better efficiency, and the Cro activity is around 20% less, all compared to those in $\lambda N^-N^-c\mathrm{I}^-hk^+$-infected bacteria. Segments of λ DNA from the left of *p*R to the right of *t*R2, carrying *cro*, cII, *O*, *P*, and the genes of the *nin*5 region from the above *hk* and hk^+ phages, were cloned in pBR322. Studies with these plasmids and their derivatives having one or more of the λ genes deleted indicate that the *hk* mutation is lethal only when a functional *P* gene is also present. When expression of *P* from *p*R is elevated, due to the deletion of *t*R1, host killing also occurs without the *hk* mutation. We conclude that the higher levels of P protein, produced either (1) when *cro* has the *hk* mutation or (2) when *t*R1 is deleted, are lethal to the host. We also show that due to the *hk* mutation, the Cro protein becomes partially defective in its negative regulation at *p*R, resulting in the expression of *P* to a lethal level even in the absence of N protein-mediated antitermination. This P protein-induced host killing depends neither on λ DNA replication nor on any other gene functions of the phage.

INTRODUCTION

In bacteriophage λ under N^- conditions, about 90% of the transcripts initiated from *p*L are terminated at *t*L1, and of the transcripts initiated from *p*R, about 60% are terminated at *t*R1 and the rest at *t*R2 (Rosenberg *et al.*, 1978; Salstrom and Szybalski, 1978; Salstrom *et al.*, 1979; Court *et al.*, 1980; Dambly-Chaudiere *et al.*, 1983). The N protein, with the help of host Nus proteins, antiterminates transcriptions at the above terminators (for a review, see Friedman *et al.*, 1987) and thus regulates positively the expression of all the delayed early and late genes of λ (Herskowitz and Hagen, 1980). Since the delayed early genes of both left and right operons of λ are needed for lytic as well as lysogenic development, λN^- neither grows lytically nor forms stable lysogens after infection of a nonpermissive host at low multiplicity, and the bacteria are not killed (Signer, 1969; Lieb, 1970). However, stable lysogens are obtained when N^- phage infects at high multiplicities, and the bacteria are not killed at all (Brooks, 1967; Chattopadhyay *et al.*, 1983). Also, the bacteria survive 100% after infection by $\lambda N^-c\mathrm{I}^-$ phage even at a superhigh multiplicity of 200, and around 20% of such infected cells form stable polylysogens carrying 25–30 copies of integrated $\lambda N^-c\mathrm{I}^-$ prophages per host chromosome (Lieb, 1971; 1972; Mandal *et al.*, 1974; Chattopadhyay and Mandal, 1982; Chattopadhyay *et al.*, 1983).

From studies with λ*clts* and λN^-*clts* lysogens, the host killing by this phage has been reported to occur in two ways after heat induction. In one, the phage *kil* gene induces such killing which is dependent on the *N* gene but not on the *O* and *P* genes of λ (Greer, 1975a). The target of the *kil* gene is a component of the host cytoplasmic membrane (Greer, 1975b). The second type of host lethality by λ has been defined by the fact that when a λN^-*clts* lysogen is heat-induced, cell killing occurs; this killing is dependent on λ prophage DNA replication promoted by the *O* and *P* gene products (Eisen *et al.*, 1968; Sly *et al.*, 1968).

While attempting to study the orientation of the 25–30 copies of integrated $\lambda N^-c\mathrm{I}^-$ prophages in the above-mentioned polylysogens, using a temperature-sensitive *Ots*26 mutation of λ isolated in our laboratory, we observed that an su^- host infected with $\lambda N^-c\mathrm{I}^-$*Ots*26 phage at an m.o.i. around 100 survived less than 0.5%, while that infected with $\lambda N^-c\mathrm{I}^-$ under identical conditions survived 100%. This interesting observation led us to study the mechanism of host killing by λ under N^- condition. The results presented in this paper show that the λ P protein, at a high intracellular level, is lethal to host. This lethality of P protein is dependent neither on any other λ gene functions nor on the DNA replication from ori^λ. Actually, in the above $\lambda N^-c\mathrm{I}^-$*Ots*26 phage, the *cro* gene is partially defective due to a mutation at its 48th codon and as a result, the efficiency of its negative regulation at *p*R is reduced; this causes an increase of total transcription from *p*R

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TABLE 1

BACTERIA AND BACTERIOPHAGE STRAINS

Strains	Alternate designation	Source
A. Bacteria (*E. coli* K12)		
(All these strains are F⁻)		
594(*galK*⁻ *galT*⁻ *lac*⁻ *thi*⁻ *Str*ᴿ *su*⁻)	594 or *su*⁻	M. Leib
594 (λ)	594(λ⁺)	Lab stock
SA500 (*Str*ᴿ *his*⁻ *relA*⁻ *su*⁻)	—	S. Adhya
DH5 (*endI*⁻ *r*⁻*m*⁺ *supII*⁺ *thi*⁻*recA*⁻ *gyr*⁻ *relA*⁻)	DH5	S. Adhya
C600(*thi*⁻ *thr*⁻ *leu*⁻ *lac*⁻ *supII*⁺)	*su*⁺ or C600	M. Lieb
DJ189[=C600(*recA*⁻ *Tl*ᴿ *galK*⁻ *Srl*::*Tn*10 *r*⁻*m*⁻)]	DJ189	D. Chattaraj
MF641(*malT*⁺ *Str*ᴿ *su*⁻ *groP*⁺)	*groP*⁺	M. Sunshine
MF639[=MF641(*gro*PA15)]	*gro*PA15	M. Sunshine
MF640[=MF641(*gro*PB558)]	*gro*PB558	M. Sunshine
MF634[=C600(*gro*PC259)]	*gro*PC259	M. Sunshine
B. Bacteriophages		
λ(wild-type)	λ⁺	M. Lieb
λ*cI*857	λ*cIts*	M. Lieb
λ*cI*857Osus905	λ*cItsO*⁻	W. Dove
λ*cI*857*Osus*1005	—	W. Dove
λ*cI*857*Osus*125	—	W. Dove
λ*cI*857*Osus*225	—	W. Dove
λ*cI*857*Ots*26	λ*cI*857*Ots*26(*hk*)	This work
λ*cIts*20*sus*29	—	M. Lieb
λ*cIts*2*Psus*3	λ*cItsP*⁻	M. Lieb
λ*N*sus7*sus*53*cI*60	λ*N*⁻*cI*⁻	M. Lieb
λ*N*sus7*sus*53*cI*60*Psus*3	λ*N*⁻*cI*⁻*P*⁻	Lab stock
λ*N*sus7*sus*53*cI*60*Osus*29	λ*N*⁻*cI*⁻*O*⁻	Lab stock
λ*N*sus7*sus*53*cI*60*Psus*3*hk*	λ*N*⁻*cI*⁻*P*⁻*hk*	This work
λ*N*sus7*sus*53*cI*60*Ots*26*hk*	λ*N*⁻*cI*⁻*Ots*26*hk*	This work
λ*N*sus7*sus*53*cI*60*Osus*905*hk*	λ*N*⁻*cI*⁻*O*⁻*hk*⁻	This work
λ*N*sus7*sus*53*cI*60*rots*2*hk*	λ*N*⁻*cI*⁻*rots*2*hk*	This work
λ*N*sus7*sus*53*cI*60*rots*6*hk*	λ*N*⁻*cI*⁻*rots*6*hk*	This work
λ*N*sus7*sus*53*cI*60*rots*19*hk*	λ*N*⁻*cI*⁻*rots*19*hk*	This work
λ*N*sus7*sus*53*cI*60*hk*	λ*N*⁻*cI*⁻*hk* or λ*N*⁻*cI*⁻*O*⁺*hk*	This work
λ*N*sus7*sus*53*cI*60*Ots*26*Psus*3*hk*	λ*N*⁻*cI*⁻*Ots*26*P*⁻*hk*	This work
λ*cI*⁺ *piA*	—	This work
λ*cI*⁺ *piB*	—	This work
λ*cI*⁺ *piC*	—	This work
λ*c*47	λ*cI*⁻	M. Lieb
λ*imm*21	—	M. Lieb
λ*imm*21*Osus*29	λ*imm*21*O*⁻	This work
λ*imm*21*Osus*905	—	This work
λ*imm*21*Ots*26	—	This work
λ*imm*434*cI*⁻	—	M. Lieb

which, in turn, elevates the expression of *P* to a lethal level even in the absence of *N*-mediated antitermination at *t*R1.

MATERIALS AND METHODS

Materials

Bacterial and bacteriophage strains are described in Table 1. Plasmids are described under Methods and in Fig. 1.

Compositions of tryptone broth, tryptone broth with maltose (TBM), phage dilution medium, and tryptone agar (TA) are described in Chattopadhyay and Mandal (1982). Ampicillin, when required, was added at 50 μg/ml prior to inoculation either in broth or on an agar plate.

Restriction enzymes and other enzymes used for recombinant DNA work were purchased from Bethesda Research Laboratories (Gaithersberg, MD); [^{14}C]ga-

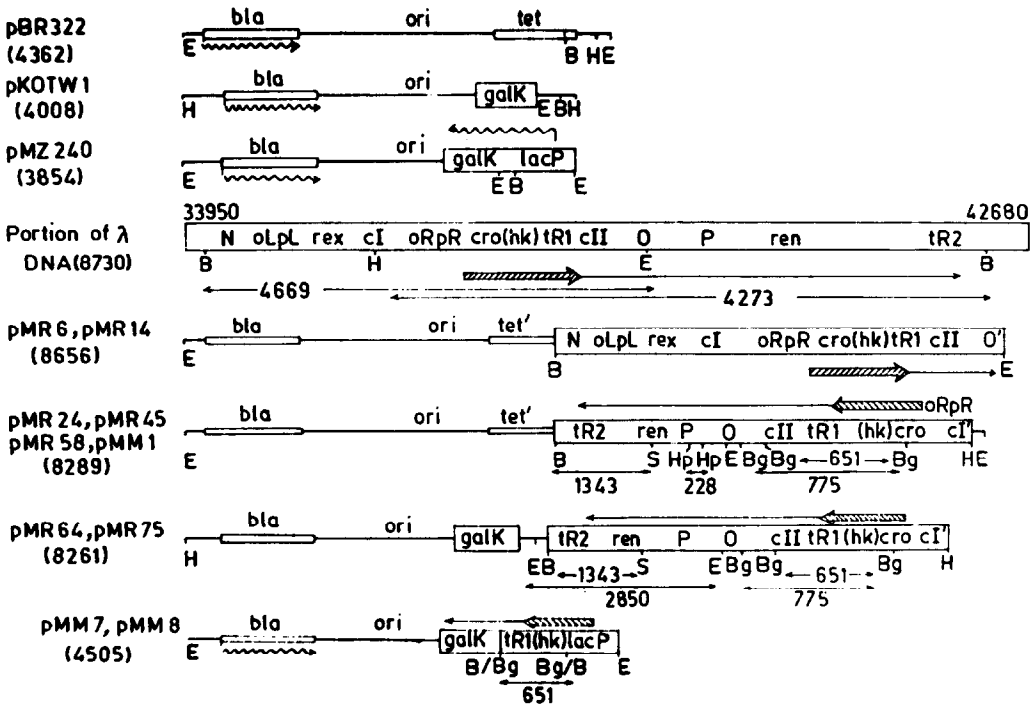

FIG. 1. Maps of different plasmids. Maps of the plasmids are shown in their linearized form. The map lengths are shown approximately proportional to their sizes. The size of a plasmid in base pairs is indicated by the number below the name of the plasmid. The map of the portion of the λ genome from 33,950- to 42,680-bp coordinates of λ DNA is shown. The restriction enzyme symbols used were as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; S, *Sst*II. The positions of restriction enzyme sites are shown by their respective symbols written below the map. The positions of different genes and regulatory sites of λ in the plasmids are not shown according to scale. The numbers indicate the base-pair lengths flanked by the two restriction sites spanned by the arrows. The combination thick and thin arrows show the directions of transcription from promoters being partially terminated at *t*R1 at the junction of the thick and thin arrows. The position of *hk* mutation is shown in parentheses next to *cro*, but this mutation actually maps within *cro* near its C-terminus. pMR6, pMR24, and pMR64 do not carry the *hk* mutation, while pMR14, pMR45, pMR58, pMR75, and pMM1 do carry it. Other details about the construction of various plasmids and their different deletion derivatives are described under Methods.

lactose was from Amersham (England); and [^{3}H]thymidine was from Bhaba Atomic Research Center (Trombay, Bombay).

Methods

Growth of bacteria. Unless otherwise stated, the bacterial cultures were routinely grown at 32–34°, and the growth was monitored by measuring the OD of the culture at 590 nm. When bacteria carrying plasmids were grown, the required antibiotic was added to the growth medium.

Preparation of bacteriophage, phage DNA, and plasmid DNA. Bacteriophage was prepared by growth and lysis in permissive host in broth and purified by PEG precipitation and CsCl banding, and the DNA was isolated, all according to the procedures described by Maniatis *et al.* (1982).

Plasmid DNA was isolated either by the lysozyme–alkaline SDS procedure of Birnboim and Doly (1979) as described by Maniatis *et al.* (1982) or by the ethidium bromide–lysozyme lysis procedure of Mukhopadhyay and Mandal (1983).

Transformation of bacteria with plasmid DNA. Transformation of bacteria with plasmid DNA was done as described by Maniatis *et al.* (1982). *Escherichia coli* 594 or any derivative thereof, when used as recipient during transformation, was grown to A_{590} of 0.9 to 1.0 and then made competent as usual. The heat shock treatment after DNA uptake was done for 30 min at 30° instead of 2 min at 43°, and the subsequent growth of the transformed bacteria was done at 30° for 2 hr.

Plasmids and their construction. Refer to Fig. 1 for maps of some of the plasmids and clues for the construction of others. Since a lethal gene of λ, whose expression was found to be negatively regulated by CI repressor at *p*R, was planned to be cloned, a DH5(λ$^+$) lysogen was always used as the recipient during transformation for selection of the clones.

Plasmids pMR6 and pMR14 contained the 4.67-kb DNA segment flanked by the *Bam*HI and *Eco*RI sites from λ*N*$^-$*cI*$^-$ and λ*N*$^-$*cI*$^-$*hk* DNAs respectively cloned between the same two restriction sites of pBR322.

Plasmids pMR24, pMR45, pMR58, and pMM1 contained the 4.27-kb DNA segments flanked by the

*Hin*dIII and *Bam*HI sites from λ*N*⁻*c*I⁻, λ*N*⁻*c*I⁻*hk*, λ*N*⁻*c*I⁻*P*⁻*hk*, and λ*N*⁻*c*I⁻*O*⁻*hk* DNAs respectively cloned between the same two sites of pBR322.

Plasmids pMR64 and pMR75 contained the 4.27-kb DNA segment flanked by the *Hin*dIII and *Bam*HI sites from λ*N*⁻*c*I⁻*P*⁻ and λ*N*⁻*c*I⁻*P*⁻*hk* DNAs respectively cloned between the same two sites of pKOTW1.

All the above plasmids were constructed as follows: The respective λ DNAs were digested with two desired restriction enzymes and ligated separately with pBR322 or pKOTW1 DNA digested with the same two enzymes. The ligated DNAs were used to transform a DH5(λ⁺) lysogen. Plasmids were isolated from the total transformants pooled from an ampicillin plate. An aliquot of this plasmid preparation was run on an agarose gel with a standard marker of 8.6-kb pMD102 DNA (Das and Mandal, 1986) also isolated under identical conditions. After the run, the putative plasmid bands (CCC-forms) of the following expected sizes were cut out from the respective gels: For pMR6 and pMR14, the 8.6-kb band; for pMR24, pMR45, pMR58, pMM1, pMR64, and pMR75, the 8.26-kb band. Then DH5(λ⁺) was transformed with the DNA eluted as above. For each plasmid, around 10 colonies were purified and plasmids were isolated. The plasmids were then linearized with suitable restriction enzymes, and the prospective plasmids were identified by their size as determined on an agarose gel and finally confirmed by restriction analysis using two or three different enzymes.

pMM2, pMM3, and pMM4 were the deletion derivatives of pMR24, pMR45, and pMR58, respectively, lacking the 775-bp *Bgl*II segment that contains the *hk*-*t*R1 region. These parent plasmids were completely digested with *Bgl*II, self-ligated at a very low concentration of DNA, and then used to transform DH5(λ⁺). The 7.5-kb plasmid was selected by its size and finally confirmed by restriction analysis.

pMM5 was an *Hpa*I deletion derivative of pMM3 removing 228 bp from the 9th through 85th codon of the *P* gene of λ. The *Hpa*I-digested pMM3 DNA was self-ligated and used to transform DH5(λ⁺). The deletion plasmid pMM5 was identified by its size and confirmed by restriction analysis.

pMM6 was derived from pMM3 by deleting the DNA segment from *ren* through *t*R2, which is flanked by the *Sst*II and *Bam*HI sites. The parent plasmid was completely digested with the two enzymes, and the larger fragment was gel-purified. The two noncompatible cohesive ends were filled by *Taq* DNA polymerase followed by blunt-end self-ligation. The ligated DNA was used to transform DH5(λ⁺), and the above deletion plasmid was purified and then confirmed by restriction analysis.

pMM7 and pMM8 contained the 651-bp *Bgl*II fragment carrying the *hk*-*t*R1 region from pMR24 and pMR45, respectively, cloned between the *lac* promoter and *gal*K at the *Bam*HI site of pMZ240. The above 651-bp fragment was purified from *Bgl*II-digested parent plasmids and then ligated with *Bam*HI-digested pMZ240. *E. coli* DJ189 was transformed with the above ligated DNA. The transformants containing the plasmid carrying the above 651-bp insert in the terminator orientation with respect to the *lac* promoter were selected as faint red colonies among the dark red ones carrying the parent pMZ240 on a *Mac*–*Gal* plate, and the proper orientation of *t*R1 was further confirmed by restriction analysis.

pMM9 and pMM10 were the deletion derivatives of pMR64 and pMR75, respectively, lacking the 775-bp *Bgl*II segment that contains the *hk*-*t*R1 region. These were constructed exactly by the procedure used for the construction of pMM2 etc. as described earlier.

pMM11 and pMM12 contained the *p*R–*t*R1–*gal*K fusion and were derived respectively from pMR64 and pMR75 by the deletion of *t*R2. The parent plasmids were digested with *Eco*RI and the gel-purified larger fragment (5.41 kb) was self-ligated. These deletion plasmids were then selected and confirmed by the usual procedure.

pMM13 was a deletion derivative of pMR64 lacking the 3.625-kb *Bgl*II–*Bam*HI segment that contains *O, P,* and *t*R2. The parent plasmid, pMR64, was digested with the above two enzymes, and the 4.65-kb fragment was gel-purified and self-ligated. The deletion plasmid pMM13 containing the *p*R–*gal*K fusion was selected and confirmed by the usual procedure.

RESULTS

Survival of nonpermissive host after infection with different λ*N*⁻*c*I⁻ phages with and without *hk* mutation

Starting from an ethyl methanesulfonate mutagenized stock of λ*c*I857, an *Ots* mutant (*Ots*26) was isolated as one of the several temperature-sensitive mutants showing delayed lysis or no lysis of the host at 42° and was confirmed by its inability to complement λ*O*⁻ phage and to synthesize phage DNA, both at 42°. Then λ*N*⁻*c*I⁻*Ots*26 was prepared by a cross of the above λ*c*I857*Ots*26 with λ*N*⁻*c*I⁻*P*⁻. One such λ*N*⁻*c*I⁻*Ots*26 recombinant was found to show multiplicity-dependent killing of an *su*⁻ host at 32° (curve 1, Fig. 2). It was further observed that around 20 revertants (reversion frequency ≈ 10^{-7}) of λ*N*⁻*c*I⁻*Ots*26 independently isolated on an *su*II⁺ host at 42° (these *r*evertants of *Ots*26 will be referred hereafter as *rots* phages) also

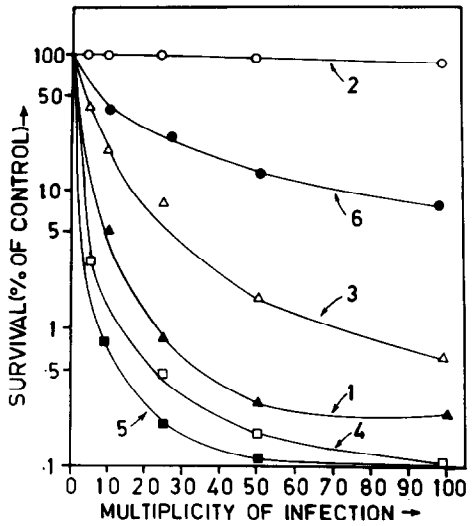

FIG. 2. Survival of bacteria after infection with λ*N*⁻cI⁻ phages with and without the *hk* mutation. Bacteria were grown in TBM to 0.6 to 0.8 OD and then mixed with desired phages at required multiplicities. After adsorption at 25–30° for 15 min, both the infected and uninfected bacteria were spread on TA plates at appropriate dilutions and incubated at 32°. After 18–20 hr, the colonies were counted. The survival of the phage-infected bacteria was calculated as the percentage of uninfected control that formed colonies under identical conditions. The host used was *E. coli*594 (*su*⁻). The different curves represent the survival after infection with the phages as follows: 1, λ*N*⁻cI⁻*Ots*26*hk*; 2, λ*N*⁻cI⁻ or λ*N*⁻cI⁻*Ots*26 (nonkiller); 3, λ*N*⁻cI⁻*rots*2*hk*; 4, λ*N*⁻cI⁻*rots*6*hk* or λ*N*⁻cI⁻*O*⁺*hk*; 5, λ*N*⁻cI *rots*19*hk*; 6, λ*N*⁻cI⁻ + λ*N*⁻cI⁻*Ots*26*hk* (1:1). The survival pattern of 594 (λ⁺) after infection with λ*N*⁻cI⁻*hk* at varying m.o.i. was exactly as represented by curve 2.

showed the killing of *su*⁻ host at both 32 and 42°. The killing results of three such *rots* phages are shown in Fig. 2 (curves 3–5). These results led us to check the host killing property of several other λ*N*⁻cI⁻*Ots*26 and λ*N*⁻cI⁻*O*⁺*P*⁺ recombinants from the above-mentioned cross, and it was found that some of the former and all of the latter were nonkillers of *su*⁻ bacteria even at 32°. These results suggest that the host killing was caused not by the *Ots*26 mutation but possibly by a second mutation in the right operon to the left of the *Ots*26 locus. By extensive phage cross-experiments, the *Ots*26 mutation was mapped in the C-terminal half of the *O* gene between *Osus*1005 and *Osus*125 (Furth *et al.*, 1978), while the killer mutation was mapped around the C-terminus of the *cro* gene (recombination data not shown; see Fig. 4 for the map positions). This latter mutation was called *hk* (for *h*ost *k*iller). The λ*N*⁻cI⁻*O*⁺*P*⁺*hk* recombinant selected at 42° on an *su*⁺ host from a cross of λ*N*⁻cI⁻*Ots*26*hk* with λ*imm*21*O*⁺*P*⁺ was found to show host killing with a profile exactly like that of λ*N*⁻cI⁻*rots*6*hk* phage (curve 4, Fig. 2). It was also observed that 594 could be partially protected from being killed by λ*N*⁻cI⁻*hk* phage when coinfected with λ*N*⁻cI⁻*hk*⁺ (curve 6, Fig. 2), and also, the former phage failed to kill a 594(λ⁺) lysogen (curve 2, Fig. 2). These results suggest respectively that the *hk* mutation is recessive to its wild-type allele in *trans* and that the host lethality of this mutation is negatively regulated by the CI repressor at *p*R of λ.

To avoid the undesirable effect(s), if any, of either the *Ots*26 mutation or its *rots* revertants on the properties of host or phage, the λ*N*⁻cI⁻*O*⁺*hk* phage obtained by the cross of λ*N*⁻cI⁻*Ots*26*hk* with λ*imm*21*O*⁺*P*⁺ as mentioned above was used in all the experiments described in the following sections.

Properties of λ*N*⁻cI⁻*hk* phage and its infected bacteria

After infection of 594 by λ*N*⁻cI⁻*hk* at an m.o.i. of 50, the level of Cro protein (measured by the determination of its operator binding activity according to the procedure of Folkmanis *et al.*, 1976) at 30 min was 16 units and that of endolysin (determined by measuring the lysis of EDTA-sensitized bacteria by the procedure of Jacob *et al.*, 1957) at 3 hr was 40 units, while those after infection with λ*N*⁻cI⁻*hk*⁺ under identical conditions were respectively 20.5 and 0 units. The phage DNA replication occurred with better efficiency after infection with the former phage compared to the latter (Fig. 3, curves 6 and 4, respectively).

After infection by the λ*N*⁻cI⁻*hk* phage, the induction of β-galactosidase was significantly depressed, and after 3 hr of postinfection growth, the cells became filamented and susceptible to lysis by chloroform or a low concentration of SDS (results not shown) while after infection by λ*N*⁻cI⁻*hk*⁺ under identical conditions, none of the above effects on the host could be observed.

Sequencing of the *hk* mutation

Both the expression of endolysin to a significant level and increased λ DNA synthesis under *N*⁻ conditions in the presence of the *hk* mutation compared to those in its absence suggest that though no antitermination mediated by the N protein at *t*R1 and *t*R2 occurs in the absence of functional *N* gene, yet the transcription initiated from *p*R could express the genes *O*, *P*, etc. beyond *t*R1 more efficiently and those beyond *t*R2 to a significant level in the presence of the *hk* mutation compared to in its absence under otherwise identical conditions.

Results of determination of the exact map position of the *hk* mutation by DNA sequencing indicate a single change of base from C to A replacing glycine by glutamic acid at the 48th codon of the *cro* gene (see Fig. 4).

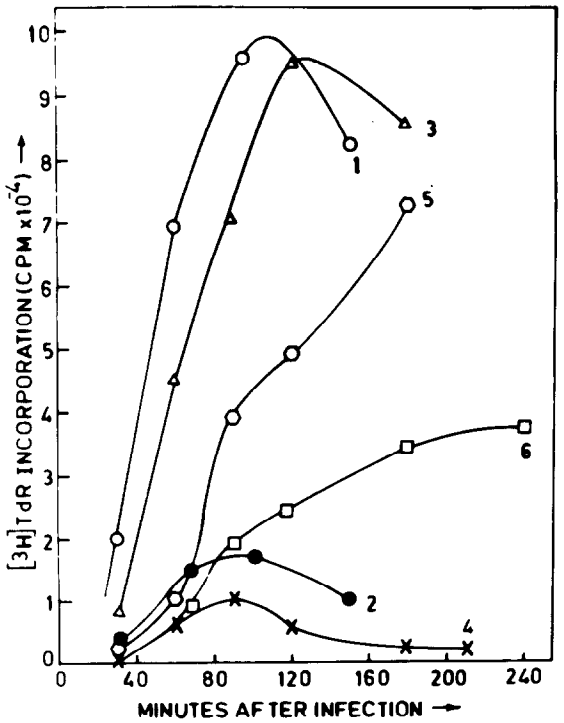

FIG. 3. The effect of the *hk* mutation on λ DNA synthesis in the absence of *N* gene function. *E. coli*594 was grown in TBM to 0.6 OD. Aliquots of this culture were infected with the desired phages at 50 m.o.i. After adsorption for 15 min at room temperature, each of the infected and uninfected cultures was diluted 2.5-fold with fresh TBM. Then to one from each duplicate set of infected and uninfected cultures was added nalidixic acid to a final concentration of 20 μg/ml (by trial experiment, this concentration of the antibiotic, at which 80–85% of the host DNA synthesis was inhibited without affecting λ DNA synthesis was chosen), while the second of each set was kept without the drug. Both the sets were incubated at 32° on a water bath shaker. At the indicated times, 0.5 ml from each was transferred into a test tube containing 1 μCi of [^{3}H] thymidine, and the tubes were shaken at 32°. After 4 min, 100 μg of cold thymidine was added and the mixture was immediately chilled on ice. Then trichloroacetic acid insoluble radioactivity was determined. The curves are as follows: 1, 594; 2, 594 + antibiotic; 3, 594 + λ*N*$^-$c$^-$; 4, 594 + λ*N*$^-$c$^-$ + antibiotic; 5, 594 + λ*N*$^-$c$^-$*hk*; 6, 594 + λ*N*$^-$c$^-$*hk* + antibiotic. The radioactivity incorporation data in the presence of nalidixic acid shown by the curves 4 and 6 represent the λ DNA synthesis after infection with λ*N*$^-$c$^-$ and λ*N*$^-$c$^-$*hk* phages, respectively.

Now, the question arises how the above mutation in *cro* could show host lethality in the absence of *N* function of λ. To identify precisely the phage-coded lethal function, the survival of an *su*$^-$ host after transformation with different plasmids carrying the *hk* mutation and other delayed early genes of λ (see Materials and Methods and Fig. 1 for details about the plasmids) were compared and the results are presented in Table 2. Both pMR6 and pMR14 carry the λ DNA segment containing *N* (*N*$^-$), *c*I (c$^-$), *rex*, *cro*, *c*II, and the left half of *O*, and the latter plasmid also carries the *hk* mutation while the former does not. It is seen that the survival of both 594 and 594(λ$^+$) after transformation with these two plasmids was nearly the same (lines 1 and 2, respectively). These results suggest that the *hk* mutation by itself does not have any host lethal property, and the putative gene which has such property is possibly not present within the DNA segment of the right operon from *cro* to the middle of the *O* gene of λ.

Since the *hk* mutation caused increased expression of the genes beyond *t*R1 and *t*R2 in the absence of *N*, we thought that possibly the primary effect of this mutation is to increase the expression of genes beyond the above terminators even in the absence of N protein, and the host killing may be a secondary effect actually caused by the product of any of the genes thus expressed at an increased level. Survival of the *su*$^-$ host after transformation with the plasmids pMR24 and pMR45, both of which carry the replication genes *O* and *P* along with the upstream *t*R1 site and a few downstream genes of λ, indicates that the former plasmid carrying no *hk* mutation did not kill the host while the latter carrying this mutation did (lines 3 and 4, Table 2). These results suggest that the putative gene responsible for the host lethality is present within the phage DNA segment carried by both the plasmids, but

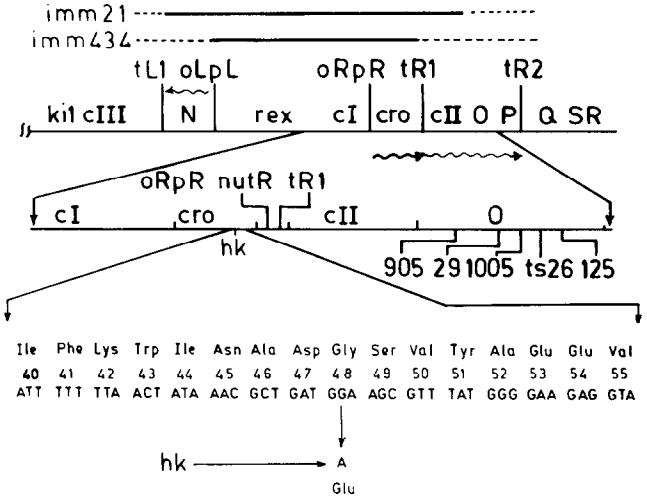

FIG. 4. Map positions of the *Ots*26 and *hk* mutations. The *Ots*26 mutation was mapped by crosses using *Osus*905, *Osus*29, *Osus*1005, *Osus*125, *Osus*205, and *Psus*3 as markers of the two parents in different combinations and determining the frequencies of *O*$^+$*P*$^+$ recombinants at 42° on *su*$^-$ host. The *hk* mutation was roughly mapped by crosses using *imm*434 and *imm*21 boundaries and *Nsus*7*sus*53, *c*I60, and *Psus*3 mutations as markers, and determining the frequencies of killer and nonkiller recombinants. The *hk* mutation could never be transferred from λ to λ*imm*434 and λ*imm*21 phages. The sequencing of DNA carrying the *hk* mutation was done using Sequenase Protocol as described by the manufacturer, U.S. Biochemical Corp., for double-stranded DNA using the plasmids pMR14 (for the *hk* mutation) and pMR6 (for *hk*$^+$ wild-type control for comparison) as templates.

TABLE 2

SURVIVAL OF *E. coli* 594 AFTER TRANSFORMATION WITH DIFFERENT PLASMIDS

Plasmid[a]	Status of λ genes in plasmids[b]							Survival of bacteria after transformation[c]	
	hk	*t*R1	*cII*	*O*	*P*	*ren*	*t*R2	594(λ⁺) (No. of transformants per μg of DNA)	594
pMR6	w	+	w	d	−	−	−	3.0×10^5	3.3×10^5
pMR14	m	+	w	d	−	−	−	1.8×10^5	2.4×10^5
pMR24	w	+	w	w	w	w	+	4.5×10^5	3.0×10^5
pMR45	m	+	w	w	w	w	+	4.5×10^5	$<10^2$
pMR58	m	+	w	w	m	w	+	4.2×10^5	3.7×10^5
pMM1	m	+	w	m	w	w	+	4.2×10^5	$<10^2$
pMM2	d	d	d	d	w	w	+	1.2×10^5	$<10^2$
pMM3	d	d	d	d	w	w	+	1.3×10^5	$<10^2$
pMM4	d	d	d	d	m	w	+	2.7×10^5	3.0×10^5
pMM5	d	d	d	d	d	w	+	5.2×10^5	3.6×10^5
pMM6	d	d	d	d	w	d	d	5.2×10^5	$<10^2$

[a] All the plasmids carried the *p*R promoter upstream of the λ genes and also had either the N^- mutation or no *N*.

[b] Gene status symbols used: w, wild-type; m, mutant-type; d, deletion by restriction cleavage (partial or complete). + sign indicates the presence of the respective terminators; − sign indicates the absence of the genes and the sites.

[c] The transformation was done using around 40 ng of DNA for each set. For each plasmid, exactly the same amounts of DNA were used and same amounts of transformed bacteria were plated on an ampicillin plate. For all the plasmid sets, the plates were incubated at 32°, and the colonies were counted after 20 hr. The lethal effect of a plasmid bearing λ gene(s) was inferred by its extreme low efficiency of transformation of 594 ($< 10^2$ PFU/μg DNA). For the other details, see Materials and Methods.

that this gene could show the killing effect in the presence of the *hk* mutation in pMR45, while in the absence of the mutation in pMR24, the same gene is unable to show this effect. To identify the lethal function, the survival of hosts after challenging with the plasmids pMR58 and pMM1 was studied. It is clear from the results in Table 2 that pMR58 carrying the *hk* mutation, *t*R1 site, functional *O*, and nonfunctional *P* did not show the lethal effect, while the plasmid pMM1 also carrying the above mutation and the *t*R1 site but with mutated *O* and wild-type *P* did show the effect (lines 5 and 6 respectively, Table 2). These results suggest that possibly the *P* gene product of λ is involved in the *hk* mutation dependent host killing.

Host lethality in the presence of the *hk* mutation in the absence of *N* function of λ is caused by increased expression of *P* and is phage DNA replication independent

In the absence of *N* function, the transcription initiated from *p*R is around 60% terminated at *t*R1 (Court *et al.*, 1980; Rosenberg *et al.*, 1978), and in the absence of the CI repressor, the initiation of this transcription is negatively regulated by the Cro repressor at *p*R (Takeda *et al.*, 1977; Takeda, 1979). The results presented in the above two sections led us to conclude that in the absence of functional *N*, the *p*R-driven transcription causes increased expression of *P* and other genes situated after *t*R1 in the presence of the *hk* mutation relative to that in its absence and possibly this increased level of P protein is lethal to host. It was further observed that though the plasmid pMR24 (structure: *p*R–*cro*(*hk*⁺)–*t*R1–O^+–P^+) did not show the killing effect, the plasmid pMM2 obtained by deleting *t*R1 and the N-terminal half of *O* from pMR24 (see Fig. 1 and Materials and Methods) was indeed very effective in showing the lethal effect (line 7, Table 2). This suggests that only the limiting expression of *P* from *p*R in pMR24 in the absence of both the *hk* mutation and *N* function in the presence of *t*R1 could not effect host killing, but when this terminator is removed from the same pMR24, the expression of *P* from *p*R was no longer limiting, and so the host killing was observed with pMM2. When both the *hk* locus and the *t*R1 site were deleted from the killer plasmid pMR45, the resulting plasmid pMM3 still showed the host killing property (line 8, Table 2). When the *P* gene had either an amber mutation or a 228-bp internal deletion respectively in pMM4 and pMM5, both these plasmids failed to show the host lethality even though both of them had the *t*R1 deletion (lines 9 and 10, Table 2). From all these results, it can be concluded that the *hk* mutation is not needed for the killing effect if the expression of func-

tional *P* is increased in the absence of the transcription barrier *t*R1. When the *ren* and other genes of the *nin*5 region were deleted from the plasmid pMM3, the resulting plasmid pMM6 still retained the host killing property (line 11, Table 2), indicating that the *P* gene could show host killing in the absence of any of the genes of the *nin*5 region of λ. The fact that *P*-mediated host killing occurs even in the absence of functional *O* gene (lines 6 and 10, Table 2) also indicates that this host lethality is not dependent on DNA synthesis from the λ origin.

Increased expression of the genes downstream of *t*R1 in the presence of the *hk* mutation is due to increase of total transcription from the *p*R promoter

Regarding the role of the *hk* mutation in increasing the expression of genes beyond *t*R1 in the absence of N protein, there are two possibilities: (i) As this mutation is located around 75 bp upstream of the *nut*R site, this may have a *cis* effect causing an increase of *N*-independent antitermination at *t*R1; or (ii) while the efficiency of termination at *t*R1 remains unchanged at 60%, the *hk* mutation makes the Cro protein functionally defective which results in an increase of total transcription initiated from *p*R. Under such conditions, 40% of the increased transcription which passes through *t*R1 (Rosenberg *et al.*, 1978; Court *et al.*, 1980) would be increased and this, in turn, would elevate the expression of replication and other genes downstream.

The first possibility was tested as follows: The plasmids pMM7 and pMM8 carry respectively the *lac*P–*hk*$^+$–*t*R1–*gal*K and *lac*P–*hk*–*t*R1–*gal*K fusions (see Fig. 1 and Materials and Methods for the details of these plasmids). In these plasmids, the expression of *gal*K would be negatively regulated by the *lac* repressor at the *lac*P promoter and would also be under *t*R1 control. So, if the *hk* mutation has *cis* effect in causing *N*-independent antitermination at *t*R1, then the level of galactokinase in pMM8-bearing cells would be higher compared to that in pMM7-carrying ones. The results in Table 3 (lines 2 and 3) show that the termination efficiency at *t*R1 both in the absence and in the presence of the *hk* mutation was the same. These results suggest that the *hk* mutation has no *cis* effect in reducing the efficiency of transcription termination at *t*R1 in the absence of *N* function.

The fact that the Cro activity in λ*N*$^-$c*I*$^-$*hk* phage-infected bacteria was around 20% less than that in λ*N*$^-$c*I*$^-$*hk*$^+$-infected bacteria supports the above-mentioned second possibility. This was confirmed as follows: The plasmids pMR64, pMR75, pMM9, pMM10, pMM11, and pMM12 all carry the *gal*K gene tagged to

TABLE 3

EFFECT OF *hk* MUTATION ON TERMINATION AT *t*R1 OF TRANSCRIPTION INITIATED FROM UPSTREAM *lac* PROMOTER[a]

Bacteria	Status of *lac*P–*gal*K fusion	Galactokinase level[b] units/A_{590}/min	Galactokinase level[b] % of control
594 (pMZ240)	*lac*P–*gal*K	154.22 ± 1.21	100.00
594 (pMM7)	*lac*P–*hk*–*t*R1–*gal*K	33.37 ± 4.36	21.63
594 (pMM8)	*lac*P–*hk*–*t*R1–*gal*K	32.91 ± 1.41	21.94

[a] Bacteria were grown overnight in minimal medium, and the next day, the culture was diluted 50-fold in the same medium containing 1 m*M* IPTG and allowed to grow at 37° with shaking to around A_{590} of 0.5. The cells were then chilled, and galactokinase was assayed by the procedure of Adhya and Miller (1979). One unit of galactokinase has been defined as the amount of activity that could produce 1 nmole of galactose 1-phosphate from free galactose under the conditions of assay.

[b] Each result represents an average of three independent experiments. For the other details, see Materials and Methods.

*p*R with the regulatory elements *cro, hk, t*R1, and *t*R2 placed between them in different combinations (for details, see Materials and Methods and Table 4). In these plasmids, the expression of *gal*K would be regulated by the Cro repressor at *p*R (Ptashne *et al.*, 1980), and in the absence of *N* function, by Rho at *t*R1 (Roberts, 1969). In the absence of *cis* effect on termination at *t*R1 by the *hk* mutation as shown above, any change in the negative regulatory property of Cro at *p*R by this killer mutation would be reflected in the levels of galactokinase in the bacteria carrying separately the above plasmids. The data on the level of this enzyme due to these plasmids in a nonlysogen are shown in the column labeled 594 in Table 4. It is seen that in the absence of *N*, the *hk* plasmid pMR75 (structure: *p*R–*cro*(*hk*)–*t*R1–*t*R2–*gal*K) produced 1.5- to 2-fold more galactokinase than the *hk*$^+$ control plasmid pMR64 (structure: *p*R–*cro*(*hk*$^+$)–*t*R1–*t*R2–*gal*K). When the *hk*–*t*R1 region, flanked by the two *Bgl*II sites, was deleted from both pMR64 and pMR75, which also removed the C-terminal part of *cro,* the galactokinase levels maintained by the resulting plasmids pMM9 and pMM10 were low and nearly the same in both (lines 4 and 5, column labeled 594). These results suggest that in the absence of the *hk* mutation, *t*R1, and functional *cro,* the termination efficiency at *t*R2 is the same, which is very high for both the deletion plasmids. When *t*R2 was deleted from both pMR64 and pMR75, the resulting plasmids pMM11 and pMM12 retained their *hk*–*t*R1 regions and also their respective *cro,* and the *gal*K gene was now linked directly to *t*R1. Under such conditions, the *gal*K expression was increased in both cases but

TABLE 4

EFFECT OF *hk* MUTATION ON TERMINATION AT *t*R1 AND *t*R2 OF TRANSCRIPTION INITIATED FROM *p*R PROMOTER[a]

Plasmid[b]	Status of λ genes and regulatory sites upstream of *galK*[c]					Galactokinase activity[d] (units/A_{590}/min)	
	pR	*cro*	*hk*	*t*R1	*t*R2	594	594 (λ112)
pKOTW1	—	—	—	—	—	4.02 ± 0.04	5.55 ± 0.25
pMR64	+	w	w	+	+	4.08 ± 0.00	3.57 ± 0.56
pMR75	+	m	m	+	+	6.11 ± 0.55	7.54 ± 1.16
pMM9	+	d	d	d	+	2.05 ± 0.35	4.60 ± 0.26
pMM10	+	d	d	d	+	1.75 ± 0.05	4.10 ± 0.30
pMM11	+	w	w	+	d	10.45 ± 0.25	13.55 ± 1.75
pMM12	+	m	m	+	d	91.42 ± 6.52	101.30 ± 3.00
pMM13	+	d	d	d	d	—	266.17 ± 12.60

[a] Termination of *p*R-driven transcription was studied by comparing the expression of *gal*K situated downstream of terminators in different plasmids.

[b] To compare the galactokinase levels under identical conditions of the plasmid copy numbers (by pBR322 origin-dependent replication only), all the pMR and pMM plasmids used contained P^- mutation by which both the lethal effect of P protein and any extra replication of plasmid from λ origin in the presence of the *hk* mutation were avoided.

[c] For the gene status symbols, see Table 2.

[d] 594-carrying plasmid was grown at 37° and the galactokinase was assayed. 594 (λ112)-carrying plasmid was grown at 32° to A_{590} of 0.4 and then induced at 41° with shaking. After 10 min, the cultures were chilled and galactokinase was assayed. For the other details, see Table 3 and Materials and Methods. Each result represents an average of three independent experiments for each bacterial set.

when the *hk* mutation was present, the level of the enzyme was around ninefold higher. When the *gal*K gene was linked directly to *p*R with no terminator between them in plasmid pMM13, the level of this enzyme could not be determined in a nonlysogen because the bacteria harboring such a plasmid without any terminator downstream did not survive. The phage λ112 is a derivative of λ*imm*21*cI*$^+$ carrying *prm*$^\lambda$–*cI*857$^\lambda$–*lac*Z fusion within the *b* region (Maurer *et al.*, 1980). When pMM13 was introduced into a 594(λ112) lysogen, the bacteria survived at 32° due to the presence of intracellular *c*I857 repressor. On heat induction at 42° and during subsequent growth at 40°, the galactokinase level increased for 10 min and then decreased steeply for unknown reasons (data not shown). So, the galactokinase synthesized by all the above-mentioned plasmids including pMM13 in 594(λ112) at 10 min after heat induction was measured. All the data are presented in the column labeled 594(λ112) in Table 4. It is seen that the enzyme levels for all the plasmids in 594(λ112) agree well with the levels for the same plasmids in the nonlysogen at 37°. These results indicate that the level of galactokinase expressed from pMM12 carrying the *p*R–*hk*–*t*R1–*gal*K fusion was about 2.6 times less than that from pMM13 carrying *p*R–*gal*K fusion. It is apparent from these results that due to the presence of the *hk* mutation in *cro*, the expression of genes situated after *t*R1 is increased seven- to ninefold over that in absence of the mutation when studied in multicopy plasmid. However, this increase of galactokinase could not reach the level that is expressed from *p*R when *t*R1 was not present between this promoter and *gal*K.

The *E. coli gro*P mutants are susceptible to *P* gene lethality

During the initiation of λ DNA replication, the P protein physically interacts with the host DNA replication proteins DnaB, DnaK, and DnaJ (Tsurimoto *et al.*, 1982; Friedman *et al.*, 1984; Dodson *et al.*, 1989; Alfano and McMacken, 1989; Liberek *et al.*, 1990). *gro*P mutants do not allow λ DNA replication, possibly due to the lack of interaction of P protein with the altered DnaB or DnaJ protein in the above-mentioned mutant hosts (Georgopoulos and Herskowitz, 1971; Georgopoulos, 1977; Sunshine *et al.*, 1977). To test whether the lethal action of the λ P protein is dependent on its interaction with the GroP components (DnaB and DnaJ) of the host, the survival of *gro*P bacteria after challenging with a lethal concentration of P protein expressed from pMR45 was studied. The results presented in Table 5 show that all three types of *gro*P mutants, *gro*PA15, *gro*PB558, and *gro*PC259, were equally susceptible to killing by λ P protein. Furthermore, it was observed that the λ*N*$^-$*cI*$^-$*hk* phage could show multiplicity-dependent killing of all the above-mentioned *gro*P *E. coli* (data not shown) and their killing profiles were similar to that of *E. coli* 594 as shown in Fig. 2 (curve 4).

Studies of [^{3}H]thymidine incorporation into host DNA in the presence and absence of functional *P* gene

TABLE 5

SURVIVAL OF *E. coli groP* MUTANTS AFTER TRANSFORMATION WITH λ *P* PLASMID pMR45

Bacteria	Transformation efficiency (No. of transformants/μg DNA)
594 (λ^+)	4.50×10^5
594	$<10^2$
*gro*P (λ^+)	4.00×10^5
*gro*P	$<10^2$
*gro*PA15 (λcI⁺*pi*A)	1.35×10^5
*gro*PA15	$<10^2$
*gro*PB558 (λcI⁺*pi*B)	1.95×10^5
*gro*PB558	$<10^2$
*gro*PC259 (λcI⁺*pi*C)	5.40×10^5
*gro*PC259	$<10^2$

Note. The λcI⁺ *pi*A and λcI⁺ *pi*B phages were isolated as spontaneous *pi* mutants by plating wild-type λ (around 10^7 PFU of λ^+ per plate) on *gro*PA15 and *gro*PB558, respectively, and selecting the plaque formers which showed up with a frequency around 10^{-5}. To isolate the λcI⁺ *pi*C mutant, wild type λ was mutagenized with hydroxylamine to a survival of 0.1% and then plated on *gro*PC259. The turbid plaque formers were purified. The lysogens of all of the above isolated λcI⁺*pi* mutants in the respective *gro*P hosts were isolated from the turbid center of the plaques and purified by the usual procedure. For transformation and other procedures, see Table 2.

with the plasmids pMR45 (structure: *p*R–*cro*(*hk*)–*t*R1–O^+–P^+), pMR58 (structure: *p*R–*cro*(*hk*)–*t*R1–O^+–P^-), and pMM1 (structure: *p*R–*cro*(*hk*)–*t*R1–O^-–P^+) in 594(λ112) after heat induction showed that under the conditions where *P* was expressed at a lethal level, the host DNA synthesis was inhibited significantly (data not shown).

DISCUSSION

In bacteriophage λ, two different types of host killing during induction of lysogens have been reported. One such lethality is effected by the phage *kil* gene, which is *N* gene-dependent (Greer, 1975a,b), while the other is induced by the initiation of DNA replication from the λ origin without excision of the integrated prophages in the absence of *N* gene function (Eisen *et al.*, 1968; Sly *et al.*, 1968). From the results presented in this paper, we now show another distinct type of host lethality of λ which involves participation of the replication gene *P* and which is not dependent on DNA replication from the phage origin.

Under normal conditions after infection by λN^- phage, the replication genes *O* and *P* are expressed to a level that is sufficient for a few cycles of replication from ori^λ (Ogawa and Tomizawa, 1968), but this low level of P protein is not lethal (Lieb, 1970, 1971; Signer, 1969; Chattopadhyay *et al.*, 1983). When the expression of *P* is elevated due to the *hk* mutation in *cro*, it is lethal to the host. This increased expression of *P* and other genes downstream of *t*R1, due to the *hk* mutation, has been shown to be an effect of increased transcription from *p*R resulting from the relaxation of negative control at this promoter by the mutant *cro*. Expression of the genes downstream of *t*R1, due to the *hk* mutation, is increased around ninefold relative to the hk^+ control (Table 4), and the elevated level of P protein expressed either in the presence of the killer mutation or in the absence of *t*R1 is lethal to the host (Table 2).

With the *hk* mutation, glycine at the 48th codon of *cro* is replaced by glutamic acid, and as a result, this repressor becomes partially defective (see result section). Pakula *et al.*, (1986) have shown that the same mutation in *cro* decreases the repressor activity to around 80% of that of the wild type, and the protein antigen becomes very unstable. Our data show that the Cro activity in λN^-cI^-*hk* phage-infected bacteria was around 78% of that in the λN^-c$I^-$$hk^+$-infected ones.

The su^- *E. coli* after infection with λN^-c$I^-$$hk^+$ phage even at the superhigh m.o.i. of 100 to 200, are not killed, and the polylysogens formed under those conditions carry 25–30 copies of integrated λN^-c$I^-$$hk^+$ prophages (Lieb, 1971; Chattopadhyay *et al.*, 1983). The level of *P* protein expressed in these polylysogens is not lethal (Lieb, 1972; Chattopadhyay and Mandal, 1982). The pBR322-derived plasmids occur in around 30 copies per cell under normal conditions of growth (Hershfield *et al.*, 1974). The plasmid pMR24 (structure: *p*R–*cro*(hk^+)–*t*R1–O^+–P^+) is a derivative of pBR322, and so this would also occur in around 30 copies per cell and would be expected to maintain an autoregulated level of the Cro protein in the carrier bacteria similar to that maintained by the 25–30 copies of derepressed λN^- prophages in the above-mentioned polylysogens (Chattopadhyay and Mandal, 1982), and hence, the level of P protein, being also regulated by the wild-type Cro in pMR24 carrier bacteria, would also be identical with that in the above polylysogens, and would not be lethal. But when the *cro* gene carries the *hk* mutation, the negative regulation at *p*R by this mutant Cro is relaxed in pMR45 (structure: *p*R–*cro*(*hk*)–*t*R1–O^+–P^+), which results in an increase of expression of *P* to a lethal level even in the absence of *N*-mediated antitermination at *t*R1 (see Results). When the terminator *t*R1 between *p*R and *P* is removed, the level of P protein is also increased and under such conditions, the *hk* mutation is not needed for P to kill the host (Table 2).

Now, the question arises as to the mechanism of host killing by the replication gene *P* of λ in the absence of its own DNA synthesis. Since all three *gro*P mutants

of *E. coli* are also susceptible to killing by the λ *P* protein (Table 5), it may be concluded that this lethality does not involve interaction of P with the host DnaB, DnaJ, or DnaK protein, which are all essential for λ DNA replication (Georgopoulos and Herskowitz, 1971; Georgopoulos, 1977; Sunshine *et al.*, 1977). The fact that the λ*N*⁻*cI*⁻*hk* phage shows m.o.i.-dependent killing of *gro*P bacteria where the killing occurs even at an m.o.i. as low as 5 or 10, indicates that the killing of *gro*P bacteria is not caused by clogging of DnaB, DnaJ, or DnaK protein by a high level of P protein made from a multicopy plasmid. The results presented in the accompanying paper (Maiti *et al.*, 1991) show that at least two types of rpl mutants, type I and type II, which survive the lethal action of λ P protein could be isolated. While the type I *rpl* mutants appear to be defective in the expression of *P* from *p*R when the *o*R is wild-type, the type II mutants seem to have acquired defect(s) in host component(s) which is possibly the target(s) of P protein-induced lethality. In the latter mutants, the growth of λ is nearly normal, which also suggests that the P-mediated host lethality does not involve interaction of this replication protein of λ with any of the host components which are essential for λ DNA replication and growth. However, the possibility of a type of interaction of the P protein other than its replicative interaction with any of those host proteins in showing the lethal effect may not be ruled out. Also, the inhibition of host DNA synthesis under the condition of high-level expression of *P* from plasmid indicates some inhibitory interaction of this protein with certain specific component(s) of the host DNA synthesis machinery. Further studies are needed to clarify these points.

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